Poly(ADP-ribose) polymerase and renal hypothermic preservation injury

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Abstract

Kidneys retrieved from cadaver donors for transplantation are preserved after harvest to attenuate the ischemic injury that occurs during the time from harvest to transplantation into the recipient. The most important preservation strategy to reduce ischemic injury is the use of simple hypothermia. Renal oxygen demand can be reduced by ~97% by simple hypothermia due largely to the Q10 effect (5). This strategy, however, is not enough to prevent preservation injury of the tissues. Hypothermia, used to reduce oxygen demand, does so in part by reducing Na-K-ATPase activity, which causes electrolyte movements down concentration gradients, resulting in dangerous cell swelling (4). Furthermore, continued hydrolysis of phosphorylated adenine nucleotides without adequate simultaneous regeneration lowers cell ATP levels and results in loss of adenine nucleotide precursor molecules at reperfusion. This leaves the cell vulnerable at reperfusion because regeneration of ATP requires adenine nucleotide precursors in the cell. To combat these unfavorable physical and chemical changes that occur during hypothermic ischemia during storage, preservation solutions are used to prevent unfavorable shifts in electrolytes and water and to maintain cellular adenine nucleotide precursor molecules for ATP synthesis at reperfusion (transplantation). These solutions contain adenosine and impermeable saccharides to prevent ATP precursor depletion and cell swelling, respectively. Thus the use of both hypothermia and preservation solutions [University of Wisconsin (UW) solution] works together to mitigate injury to kidneys during prolonged cold storage. Despite these preservation attempts, prolonged cold preservation of kidneys leads inevitably to injury from hypothermia and ischemia. The latter causes reperfusion injury at transplantation, similar to conventional reperfusion injury suffered with normothermic ischemia and reoxygenation, except that the ischemia times are longer during hypothermia due to slowed metabolism at reduced temperatures.

Tissue ischemia and reperfusion result in activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP). This enzyme is activated 500-fold by single-strand DNA breaks and cleaves NAD\(^+\) to attach ADP-ribose polymers onto proteins associated with the damaged DNA (10). This normal protective response to DNA breaks becomes excessive under conditions of severe oxidant stress as may be encountered with reperfusion of ischemic tissues and leads to depletion of ADP-ribose precursors (NAD\(^+\)). The loss of cellular NAD\(^+\) stores prevents formation of reducing equivalents for electron transfer at reperfusion, resulting in further ATP depletion and cell death. This scenario has been described as the PARP suicide hypothesis (6).

Numerous studies have demonstrated a significant role for PARP in reperfusion injury in a variety of tissues, organs, and models. Polymers of ADP-ribose appear in tissues undergoing oxidant stress and reperfusion injury. Pharmacological inhibitors of PARP significantly attenuate oxidant injury caused by reperfusion, hemorrhagic shock, stroke, sepsis, and inflammation (1, 9, 19–21, 27, 29, 31, 33). Furthermore, mice deficient in PARP through genetic knockout show significantly less severe signs of reperfusion injury in many models of inflammation and reperfusion (27). These data suggest a strong mechanistic connection between PARP overactivation at reperfusion and the devastating phenotype characteristic of reperfusion injury. Furthermore, these studies suggest that pharmacological manipulation of PARP activity may produce salutary effects in clinical settings where reperfusion injury is problematic, such as reperfusion of cold-stored organs at transplantation. Therefore, we tested the hypothesis that PARP activation occurs with cold storage of preserved kidneys and that this...
activity may produce functional degradation at reperfusion. We further attempted to lay the foundation for the rational pharmacological manipulation of PARP to treat clinical renal preservation injury.

METHODS

Animals. Renal tissue was obtained from 29 adult beagles weighing 10–12 kg, which were undergoing staged contralateral nephrectomy for renal transplantation experiments for other studies. All animals were under a surgical plane of general anesthesia that was induced with pentobarbital sodium (25 mg/kg iv) and maintained with 2% isoflurane with 50% O2. The right kidney was removed through a midline incision by careful atraumatic isolation of the blood vessels either 100 ml of UW solution (Viaspan) or lactated Ringer solution (LR). After being flushed, the kidneys were either processed for tissue slices (control tissue) or were left in cold storage at 4°C. In some experiments, the kidneys were first subjected to warm renal ischemia for 30 or 60 min before being harvested and flushed, by clamping the renal artery and vein with a vascular clamp. The animals’ core temperature was maintained at 37–38°C by using water-jacketed heating blankets at all times.

Tissue preparation. Tissue slices of the renal cortex and medulla were prepared using a Stadie-Riggs microtome to give a uniform thickness of ∼0.5 mm. Slices were prepared from kidneys cold stored for various times in UW solution. The slices were kept in Kreo-Henseleit buffer (KHB) at 4°C for ∼10 min before the time of tissue incubation or reperfusion. The tissue slices were then incubated in 3.0 ml KHB in 25-ml Erlenmeyer flasks in a Dubanoff-style metabolic incubator under an atmosphere of 95% O2-5% CO2 at 37°C for 30–240 min with oscillations to simulate reperfusion. Generally, reperfusion was for 30 min with tissue slices except for a reperfusion kinetic experiment with variable times and 120 min for isolated renal tubules. After the incubation period, the tissue slices, including those without reperfusion, were harvested, weighed, and frozen for subsequent analysis of tissue PARP activity.

Design. The effects of prolonged cold storage times and reperfusion on renal tissue PARP activity were tested in kidneys flushed with an optimum preservation solution. Kidneys were flushed at retrieval with UW solution and cold stored for 24–120 h. Tissue slices of the cortex were prepared at days 0, 1, 3, and 5 and incubated under warm oxygenated conditions in KHB for 30 min to simulate short-term reperfusion at transplantation. After reperfusion, the tissues were harvested and frozen for later determination of tissue PARP activity. Cold-stored tissue slices, without in vitro reperfusion, were also saved for PARP analysis. In some experiments in this group, the kidneys were harvested after a 30- or 60-min period of warm renal ischemia in situ by clamping the renal artery and vein. This simulates organ donation from non-beating-heart donors. These kidneys were processed as described before for kidneys without prior warm ischemia.

The effects of time-independent preservation injury on renal tissue PARP activity were determined by using two different flush solutions of different quality. Kidneys were harvested and flushed with either UW solution (good preservative) or LR solution (poor preservative). The kidneys were then subjected to ischemia for 30 min in KHB containing 3 mM H2O2, 100 μM MTT, 1 mM PMFS, and 50 μl of protease inhibitor cocktail (P-8340, Sigma), pH 8.0, using a tissue homogenizer (TR-10, Tekmar, Cincinnati, OH) on the medium power setting for 10 s. This homogenate was strained through a 75-μm mesh screen and centrifuged at 1,000 g for 5 min. The supernatant was discarded, and the pellet was reconstituted in 10 ml of the above buffer, vortex mixed, and allowed to stand undisturbed for 45 min at 4°C to sediment the debris. This supernatant was carefully removed and centrifuged at 3,000 g for 15 min. To this pellet was added 0.5 ml of PARP assay buffer containing 56 mM HEPES, 28 mM KCl, 28 mM NaCl, 2 mM MgCl2, 125 mM Na2PO4, and 0.01% wt/vol digitonin, pH 7.5. The pellets were sonicated at 50% power for 20 s (model 300 sonic dismembrator, Fisher), and the mixture was transferred to 1.5-ml conical polypropylene vials. Titrated-labeled NAD+ (50 nCi, Amersham) was added to the vials, and they were incubated for 20 min at 37°C in a gyrating water bath. The reactions were quenched with the addition of 250 μl of an ice-cold 50% TCA solution. The vials were allowed to stand for 4 h at 4°C, and the insoluble precipitate was then pelleted in a microcentrifuge at 12,000 g for 5 min. The supernatant was removed, and the insoluble pellet containing radiolabeled ADP-ribosyl polymers was washed twice more with 1-ml volumes of 5% TCA. The washed pellets were solubilized by incubating them in 1.0 N NaOH containing 2% SDS overnight at 50°C. The radioactivity was then counted in a liquid scintillation counter (Packard Tri-Carb, 1900CA, Redmond, WA) in a scintillation cocktail for 5 min each. As a control, some incubations contained the PARP inhibitors PJ34 and 3-AB. The inhibitory effect of ADP-ribose polymerization was assumed to represent specific PARP-derived synthesis.

PARP Western blot analysis. The amount of PARP protein in tissue slices during some experiments was assayed by Western immunoblotting. About 100 mg of frozen renal cortical slices were homogenized in 2 ml of H12K buffer. About 200 μl of this suspension was subjected to electrophoresis in a 7.5% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250, then subjected to electrophoretic transfer to nitrocellulose paper. The nitrocellulose paper was probed with rabbit antibody to PARP, and then incubated with β-actin antibody. The protein bands were visualized by addition of EnVision polymerized horseradish peroxidase conjugated to anti-rabbit antibody, and finally by addition of 3,3′-diaminobenzidine tetrahydrochloride as a chromogen.

PARP activity assay. About 250 mg of frozen renal tissue slices were homogenized in 5 ml of cold buffer consisting of 50 mM Tris, 25 mM MgCl2, 100 μM 2-ME, 20 μM DTT, 1 mM PMFS, and 50 μl of protease inhibitor cocktail (P-8340, Sigma), pH 8.0, using a tissue homogenizer (TR-10, Tekmar, Cincinnati, OH) on the medium power setting for 10 s. This homogenate was strained through a 75-μm mesh screen and centrifuged at 1,000 g for 5 min. The supernatant was discarded, and the pellet was reconstituted in 10 ml of the above buffer, vortex mixed, and allowed to stand undisturbed for 45 min at 4°C to sediment the debris. This supernatant was carefully removed and centrifuged at 3,000 g for 15 min. To this pellet was added 0.5 ml of PARP assay buffer containing 56 mM HEPES, 28 mM KCl, 28 mM NaCl, 2 mM MgCl2, 125 mM Na2PO4, and 0.01% wt/vol digitonin, pH 7.5. The pellets were sonicated at 50% power for 20 s (model 300 sonic dismembrator, Fisher), and the mixture was transferred to 1.5-ml conical polypropylene vials. Titrated-labeled NAD+ (50 nCi, Amersham) was added to the vials, and they were incubated for 20 min at 37°C in a gyrating water bath. The reactions were quenched with the addition of 250 μl of an ice-cold 50% TCA solution. The vials were allowed to stand for 4 h at 4°C, and the insoluble precipitate was then pelleted in a microcentrifuge at 12,000 g for 5 min. The supernatant was removed, and the insoluble pellet containing radiolabeled ADP-ribosyl polymers was washed twice more with 1-ml volumes of 5% TCA. The washed pellets were solubilized by incubating them in 1.0 N NaOH containing 2% SDS overnight at 50°C. The radioactivity was then counted in a liquid scintillation counter (Packard Tri-Carb, 1900CA, Redmond, WA) in a scintillation cocktail for 5 min each. As a control, some incubations contained the PARP inhibitors PJ34 and 3-AB. The inhibitory effect of ADP-ribose polymerization was assumed to represent specific PARP-derived synthesis.
for 20 s in 0.5 ml of extraction buffer (Tissue-PE LB, Genotech, St. Louis, MO) containing 1% protease inhibitor cocktail (P-8340, Sigma) using a PowerGen 35 homogenizer (Fisher). After homogenization, equal quantities of protein (~20 μg) were loaded onto precast minigels composed of 10% polyacrylamide in Tris-glycine-SDS (Bio-Rad, Hercules, CA). Electrophoresis was run at 20 mA/gel in Tris-glycine-SDS buffer, and the proteins were electrothermally transferred onto PVDF membranes (Bio-Rad) using a semidry transfer apparatus (Trans-Blot SD cell, Bio-Rad) operating at a 10-V constant for 75 min with Duan transfer buffer (10% methanol and 0.02% SDS). After transfer, the membranes were blocked in 5% milk proteins (Blotto-B, Santa Cruz Biotechnology, Santa Cruz, CA) in TBS-Tween 20 (0.05%) for 30 min and incubated with the primary antibody for 18 h at 4°C in Blotto-B-TBS with 0.05% Tween 20. The primary antibody was mouse monoclonal anti-human PARP (F-2 clone, Santa Cruz Biotechnology) used at a titer of 1:500. The secondary antibody was a goat anti-mouse IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnology) and was used at a titer of 1:500. After stringent washing, enhanced chemiluminescence was used to visualize the specific target bands at 112 and 85 kDa with a luminol reagent (Santa Cruz Biotechnology) and a Kodac 440 CL chemiluminescent imaging station (Kodak, Rochester, NY). Bands were quantitated by digitalized densitometry using Kodak ID imaging analysis software.

**RESULTS**

The PARP inhibitors PJ34 and 3-AB significantly suppressed PARP activity measured from normal renal cortex in preliminary experiments (Fig. 1). These data suggest that the PARP activity, as measured in this study, is attributable to the enzymatic activity of PARP-1.

A main objective of this study was to determine whether renal PARP activity changes with cold storage and reperfusion that occurs with renal transplant preservation injury. These effects are shown in Fig. 2. Renal cortical PARP enzyme activity increases with cold-storage time but only after the tissues are reperfused. Cold storage without reperfusion does not increase PARP activity. Also, the cold storage/reperfusion-induced increase in PARP activity is abrogated when the tissues undergo prior warm ischemia, as often occurs in clinical kidney transplantation. The inclusion of 3-AB, a known PARP-1 inhibitor, in the flush solution blocked the reperfusion-induced increase in labeled NAD+, suggesting the increases were due to PARP-1 enzymatic activity.

The expression of PARP-1 protein in tissue slices during cold storage and reperfusion with specific immunoblotting techniques indicates an increase in PARP-1 protein levels after progressive cold storage (Fig. 3). However, these levels were not changed after reperfusion. The antibody used to probe the blots reacted with two distinct proteins with molecular masses of 112 and 85 kDa. These probably represent the native PARP-1 enzyme and its degradation product from caspase-3 activation, respectively (14).

Figure 4 demonstrates that PARP activity in cold-stored renal tissue may be dependent on the degree of preservation stress because PARP increased after reperfusion after 24 h of cold storage only in kidneys flushed with LR solution compared with UW-flushed kidneys.
The kinetics of PARP activity during reperfusion of cold-stored renal cortex after cold storage in UW solution are shown in Fig. 5. Reperfusion resulted in increased PARP activity that peaks at 1 h after reperfusion, and the activity remained elevated but stable thereafter. This pattern was not affected by the reperfusion medium because reperfusion in Krebs buffer or whole canine blood produced similar results.

Oxidant stress has been implicated as a trigger for PARP activity. The effects of oxidant stress on normal renal tissue PARP activity are shown in Fig. 6. Hydrogen peroxide stimulated PARP activity in normal renal cortex but not in the medulla. The effect in the cortex was maximal by 1 h after addition of peroxide and remained stable for hours afterward. Although hydrogen peroxide did not activate PARP activity in the renal medulla, the basal levels of PARP activity were significantly higher (5-fold) relative to the renal cortex.

Figure 7 demonstrates the possible involvement of ROS in mediating reperfusion-induced increases in renal cortical PARP activity after cold storage. NADH utilization after 72 h of cold storage in UW solution and reperfusion was unaffected by chemical agents that are known to inhibit ROS activity (Fig. 7A). The production of 8-isoprostane, a stable byproduct of lipid peroxidation, by renal slices treated with ROS agents is shown in Fig. 7B. Chemical agents that scavenge ROS during reperfusion did not significantly alter tissue isoprostane synthesis, although an inhibitory trend was clearly observed with catalase, suggesting that renal cortical hydrogen peroxides may increase with reperfusion after cold storage in this model.

The functional significance of PARP activity in renal proximal tubules during cold preservation and reperfusion was tested in a cell survival experiment, and the results are shown in Figs. 8 and 9. Isolated canine renal proximal tubules lost ~50% viability when they were subjected to cold storage in UW solution for 48 h followed by reperfusion in vitro for 2 h, as assessed by LDH release. This represents a 3.4-fold increase in LDH release relative to either fresh tubules or tubules after cold storage but before reperfusion (44% LDH release after reperfusion vs. 13% LDH release before reperfusion and in fresh tubules). Treatment of the proximal tubules with selective PARP-1 inhibitors significantly increased the LDH release at reperfusion compared with the untreated tubules (44% untreated vs. 68% PJ34 treated), suggesting that PARP activity is beneficial during reperfusion after hypothermic cold storage in UW solution. The same tubules demonstrated a significant decrease in ATP concentration after cold storage and reperfusion in the PARP inhibitor-treated tubules, relative to tubules without PARP-1 inhibitor treatment (Fig. 8B). Intracellular ATP concentrations were 3.7 nM/mg protein after reperfusion in the untreated controls compared with 0.43 and 0.89 nM/mg protein in tubules treated with the PARP-1 inhibitors PJ34 and 3-AB, respectively.

The viability results obtained using isolated canine tubules and PARP inhibitors (PJ34 and 3-AB) were checked against a mouse strain with a genetic deficiency in the PARP-1 gene (Fig. 9). In this setting, PARP-1 genetic ablation did not alter the degree of tubule cell death after cold storage and reperfusion, nor did this maneuver potentiate the reperfusion injury observed in the PARP-1 inhibitor-treated canine proximal tubules (Fig. 8).

Isolated canine proximal tubule caspase-3 activity, a marker for apoptosis, is shown in Table 1. Neither reperfusion after cold storage nor PARP inhibition with PJ34 caused increases in caspase-3 activity. In fact, caspase activity significantly declined after cold storage and reperfusion, relative to fresh tubules.

DISCUSSION

This study focuses on renal tissue PARP activity that changes with hypothermic ischemia and reperfusion, which are
biological conditions that are associated with human renal hypothermic preservation injury after transplantation. PARP enzymes that produce poly(ADP-ribose) polymers include PARP-1, PARP-2, S-PARP, tankyrase, V-PARP, and others (7, 27). However, PARP-1 and PARP-2 are characterized by DNA repair after reperfusion, whereas the other forms of PARP are not (13). Of these, PARP-1 appears to be important in normothermic reperfusion injury and, therefore, was studied in this model. We used an NAD⁺/H⁺ consumption assay to measure PARP activity because PARPs use NAD⁺ as a precursor molecule. The PARP enzyme activity measured in this study likely reflects enzymatic activity catalyzed by the PARP-1 isozyme, which is responsible for PARP synthesis in other forms of reperfusion injury. About 25% of the NAD⁺ measured by these assays is not attributable to specific PARP-1 enzymatic activity and may be due to nonspecific background of the assay, PARP synthesis by enzymes other than PARP-1, or both.

The conditions that must be satisfied to demonstrate a role for PARP-1 in renal cold storage injury include demonstrating that enzymatic activity or its product changes during the course of preservation; demonstrating that the effects are selective and the product is inhibitable by selective antagonists; and showing that a functional or biochemical marker of preservation injury is affected by the formation or inhibition of the product; i.e., it is not an epiphenomenon of preservation injury. All three of these conditions have been fulfilled. Figure 2 clearly demonstrates that PARP enzymatic activity is increased with increased preservation times relative to the activity observed without cold storage. Furthermore, the effect is inhibited by selective inhibitors of PARP added either to the preservation solution (Fig. 2) or to the tissue in vitro (Fig. 1). The enhanced PARP activity with prolonged cold ischemia times is parallel to and consistent with the large number of reports that demonstrate increased PARP activity in models of warm ischemia-reperfusion in numerous models and tissues including the

Fig. 3. Renal tissue PARP protein expression by Western blot analysis after various cold storage times in UW solution (0–5 days) before and after reperfusion in vitro. The antibody detected both the parent enzyme (112 kDa) and the caspase-3-catalyzed hydrolysis product (85 kDa). CS, cold stored only; R, reperfused after cold storage; d, day(s); n = 3.
kidney (20, 29, 31). This is the first time, to our knowledge, that PARP enzymatic activity has been demonstrated to increase in response to hypothermic ischemia and reperfusion, although functional effects of PARP synthesis inhibitors have been observed in the cold-stored rat heart after warm reperfusion at transplantation (12, 30). Therefore, this study has demonstrated increased specific PARP-1 enzymatic activity during cold storage and reperfusion in a clinically relevant model of renal transplant preservation injury.

The current study has established that reperfusion is necessary for enhanced PARP synthesis, and this suggests that ischemia may be important because the intensity of reperfusion injury, and presumably PARP enzyme activity, depends on the length of the preceding ischemic period. However, the reperfusion-induced PARP synthesis is not totally dependent on the length of time of prior cold ischemia per se because less ischemia using a poor preservation solution (LR; Fig. 4) produces similar increases in tissue PARP activity compared with longer periods of cold ischemia using a better preservation solution (UW; Fig. 2). In fact, the reperfusion-induced PARP activity was more robust after 1 day of cold storage in LR compared with 5 days in UW solution. The different outcomes induced by these solutions for any constant cold ischemia time may be attributable to cell and tissue damage from cell swelling, acidosis, or energy failure at reperfusion. These are more pronounced in LR-preserved organs compared with organs cold stored with UW solution because the former solution is deficient in cell impermeants, buffers, and adenine nucleotide precursors, which are necessary to protect organs and tissues from hypothermic ischemia. Although the PARP activity is greater with poorer preservation solutions at shorter ischemia times, the effects are still only realized at reperfusion (Fig. 4), as occurs with optimally preserved kidneys flushed with the

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Fig. 4. Renal cortex tissue slice PARP enzyme activity in kidneys flushed with lactated Ringer (LR; A) or UW solution (B). Kidneys were stored for 24 h, and the tissue slices were analyzed before and after reperfusion in vitro in Krebs-Henseleit buffer with oxygen to simulate reperfusion after transplantation. *P < 0.05 relative to before reperfusion and for the corresponding value at day 0, n = 6.

Fig. 5. Effects of reperfusion time and reperfusion media on renal cortical tissue slice PARP enzyme activity. Tissue slices were prepared from canine kidneys flushed with UW solution and cold stored for 3 days, which is the earliest storage time required for PARP activation after 30 min of reperfusion (Fig 2). KHB, Krebs-Henseleit buffer; n = 4.

Fig. 6. Renal tissue slice PARP enzyme activity in response to oxidant stress (3 μM H2O2). Tissue slices were prepared from cortex (A) and medulla (B) from normal fresh canine kidneys. *P < 0.05 relative to time 0 control; n = 4.
UW solution. Therefore, factors that mitigate the cold ischemic damage during cold storage attenuate the reperfusion-induced increase in PARP synthesis.

The increased NAD$^+$ consumption (PARP activity) with reperfusion of cold-stored renal cortical tissue may be attributable to increased specific activity of existing enzyme, expression of more enzyme, or a combination of the two. Protein extracts from renal cortical slices subjected to hypothermic storage and reperfusion show an increase in PARP-1-specific protein in the tissue as ischemia times increase (Fig. 3). Although the PARP-1 protein levels rose during cold-storage ischemia, the protein concentrations were not further altered by subsequent reperfusion. Thus it seems that the increased PARP-1 enzymatic activity during reperfusion after prolonged cold storage may be due to stimulation of PARP-1 protein during the hypothermic ischemia period, followed by activation of the enzyme by a component associated with reperfusion. It is interesting that PARP-1 protein synthesis proceeds over time even at very low temperatures, and the activation of this protein requires an event that occurs with reperfusion. The stimuli for activation of PARP-1 protein expression during cold ischemia and the stimuli for activating the enzyme at normothermic reperfusion are not known from this study, but they probably arise from different mechanisms.

The reperfusion-induced increase in PARP-1 enzymatic activity is abrogated when a brief period of warm ischemia is induced before cold-storage ischemia and reperfusion (Fig. 2). This observation was unexpected because prior warm ischemia exacerbates renal hypothermic preservation injury, as assessed by declines in renal function at reperfusion, ATP levels, and 8-iso-prostane production. Some conditions were treated with the PARP-selective inhibitors PJ34 and 3-aminobenzamide (3-AB) during cold storage and reperfusion to detect the functional effect of PARP in cold storage reperfusion injury. EOP, end of preservation (before reperfusion). *P < 0.05 relative to control (A) and treated groups (B); n = 6.

Fig. 7. Renal tissue slice PARP activity (NAD$^+$ consumption; A) and 8-isoprostane production (B) obtained from kidneys cold stored for 72 h in UW solution and subjected to in vitro reperfusion. Renal tissue slices were reperfused in the presence or absence of chemical agents that inhibit radical oxygen species (ROS) production. Tissue isoprostanes (B) were used to indicate previous lipid peroxidation secondary to ROS stress after reperfusion; n = 5. cpm, Counts/min. *P < 0.05.

Fig. 8. Lactate dehydrogenase release (LDH; A) and cellular ATP content (B) from isolated canine renal proximal tubules subjected to cold storage in UW solution (48 h) and reperfusion (2 h) in Weinberg's solution A under an oxygen atmosphere. Some conditions were treated with the PARP-selective inhibitors PJ34 and 3-aminobenzamide (3-AB) during cold storage and reperfusion to detect the functional effect of PARP in cold storage reperfusion injury. EOP, end of preservation (before reperfusion). *P < 0.05 relative to control (A) and treated groups (B); n = 6.

Fig. 9. LDH release from isolated mouse renal proximal tubules obtained from wild-type mice with intact PARP-1 enzymes (+/+), mice with homologous deletion of the PARP-1 gene (+/−), and wild-type mice treated with the putative selective PARP-1 inhibitor PJ34. Tubules were subjected to cold storage in UW solution (48 h) and reperfusion for 2 h in oxygenated Weinberg's solution A (Reflow).
PARP activation leading to cellular necrosis states that geno-
fusion after hypothermic storage ischemia is not clearly
and reperfusion occur. This cannot be demonstrated or
response of the prior warm ischemia, before hypothermic ische-
(20). The ameliorating effect of prior warm ischemia on PARP
reperfusion, and inhibition of PARP-1 improves renal function
ischemia in rats induces PARP activity in the tissue after
warm ischemia exposure (3, 15, 16). Furthermore, warm renal
molecules, resulting in catastrophic energy collapse and necro-
sis (6). Indeed, normothermic reperfusion injury has been
from this study of signi
activity increases in cold-stored renal cortex with reperfusion in oxygenated
buffer, but reperfusion with blood containing ROS-generating
cellular components does not further alter these levels. This
suggests that circulating cellular components, either directly or
indirectly, may have no further effect on PARP activity at
reperfusion in vivo. Furthermore, although exogenous oxidants
like hydrogen peroxide stimulate PARP activity in this model
(Fig. 6), scavenging ROS with catalase, Trolox, or DMSO does
not alter the postreperfusion PARP activity (Fig. 7). In fact,
ROS are probably not produced in great quantities after reper-
fusion of hypothermically preserved kidneys because 8-iso-
prostane synthesizes in these tissues are not significantly altered
after reperfusion by ROS-scavenging agents. PARP enzymatic
activity, however, is increased without appreciable free radical
production. In fact, the role of ROS in reperfusion injury of
cold-stored organs for transplantation is dubious, as evidenced
by the lack of use of ROS-mitigating pharmacological agents
for reperfusion injury after clinical and experimental transplan-
tation (23, 24, 28). Therefore, oxidant stress is a sufficient but
not a necessary or a likely condition for activating PARP in
cold-stored kidneys after reperfusion. Some other mechanisms
elaborated at reperfusion besides blood components or ROS
generation are involved in the activation of PARP in this
model. This is contrary to predictions by the PARP suicide
hypothesis and different from reperfusion after warm ischemia.

Reperfusion injury in other organs and tissues has been shown to activate PARP, precipitate energy collapse, and cause
necrosis. Consistent with these observations is the salutary
effect of PARP inhibitors administered to animals, cells, or
tissues at the time of reperfusion (31). This does not occur with
reperfusion of hypothermic kidneys from large animals (ca-
nines) because the PARP inhibitors PJ34 and 3-AB did not
rescue isolated canine renal proximal tubules from reperfusion
injury after prolonged cold storage in UW solution (Fig. 8). In
fact, both PARP inhibitors significantly potentiated cell death in
reperfused proximal tubules, suggesting that the augmented
endogenous PARP activity at reperfusion is protective. These
cell experiments are so far supported by preliminary data using
PARP-1 inhibitors in canine kidney transplantation studies
(Mangino MJ, unpublished observations). The mechanism for
this protective role of PARP activation in organ preservation
of canine kidneys is not known but may depend on the kinetics
of PARP activity. PARP in warm ischemia models runs excess-
vously to exhaust NAD⁺ and collapse the energy charge of the
cell. PARP activity during hypothermic ischemia, although
ellevated, may run more slowly after reperfusion compared
with normothermic ischemia, thus protecting NAD⁺ levels
after reperfusion and preventing energy collapse. Furthermore,
these cells may actually utilize ADP-ribose polymers produced
by PARP to regenerate ATP during reperfusion to further
contribute to the ATP pool. This mechanism in fact occurs in
cells with damaged DNA (22) and is supported by observations
from this study of significantly higher ATP levels (4-fold) in
cold-stored tubules reperfused without PARP inhibitors com-
pared with those with PARP inhibitors (Fig. 8). The inhibition
of PARP in this setting may have prevented the generation of
ATP during reperfusion from ADP-ribose polymers catalyzed
by PARP, thus resulting in lower energy stores at reperfusion,
where ATP regeneration is already compromised by many
factors. In effect, PARP activity may channel ATP from
NAD⁺ stores. As long as the consumption of NAD⁺ for
this purpose doesn’t compromise the availability of reducing equiv-
ance for oxidative phosphorylation to produce de novo ATP,
then this mechanism may contribute to the maintenance of
cellular ATP and the attendant benefits observed during reper-
fusion without PARP inhibitors in this study.

The ability of reperfused canine renal tubules in the control
untreated group to maintain normal ATP levels despite a 50%
cell kill, as assessed by LDH release (Fig. 8), indicates that half
the cell numbers recovered from cold storage are producing
twice the amount of ATP. This is a phenomenon of the UW

![Fig. 10. Proposed effect of temperature (TEMP) on the different outcomes of PARP activation during reperfusion. In the warm ischemia-reperfusion envi-
ronment, the large amount of ROS-induced PARP activation causes massive
PARP activity, NAD⁺ depletion, and energy collapse. During hypothermic
ischemia and subsequent reperfusion, ROS stress is much less, leading to
moderate activation of PARP, which promotes genomic stability without the
deleterious effects of NAD⁺ depletion and energy collapse. This model
predicts salutary effects of PARP inhibitors in warm ischemia-reperfusion
and deleterious effects of PARP inhibitors on reperfusion injury after hypothermic
ischemia characteristic of organ preservation injury.](http://ajprenal.physiology.org/)

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**Table 1. Caspase-3 activity from canine proximal tubules after cold storage and rewarmin**

<table>
<thead>
<tr>
<th>Cold Storage Time</th>
<th>Non-treated</th>
<th>PJ34-Treated</th>
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<tr>
<td>Day 0</td>
<td>25.1 ± 3.1*</td>
<td>11.4 ± 3.3</td>
</tr>
<tr>
<td>Day 1</td>
<td>14.2 ± 1.8</td>
<td>13.7 ± 3.4</td>
</tr>
<tr>
<td>Day 3</td>
<td>13.7 ± 3.4</td>
<td>12.6 ± 1.7</td>
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Values are means ± SD of p-nitroaniline accumulation expressed as micromolar per hour per milligram protein; n = 5. *P < 0.05 relative to all values.
solution, which provides cells with high organic phosphate and adenosine concentrations during cold storage and at reperfusion. At reperfusion, the availability of both molecular oxygen and abundant adenine nucleotide precursors stimulate ATP resynthesis at very high rates (2).

Another mechanism for salutary PARP activation in hypothermic organ preservation in this model compared with normothermic reperfusion in other models may also be related to temperature and PARP activity but may depend on the normal functions of PARP in protecting the cell from genotoxic disruption. PARP may produce positive effects on DNA and tissue repair during reperfusion after both hypothermic and normothermic ischemia temperatures. At normothermic ischemia temperatures, however, PARP activity at reperfusion also runs more quickly and excessively consumes cellular reducing equivalents (NAD⁺), resulting in energy collapse characteristic of normothermic reperfusion necrosis (suicide hypothesis) and thereby offsetting the protective effects of PARP on DNA and cellular repair. This may be related to the amount of oxidant injury produced during normothermic vs. hypothermic ischemia temperatures because ROS are produced in kidneys with reperfusion after ischemia at 37°C (25) but not appreciably at hypothermic temperatures (Fig. 7). This causes more genotoxic stimuli (ROS) and hence more PARP activation at warm hypothermic temperatures because ROS are produced in kidneys with reperfusion after ischemia at 37°C (25) but not appreciably at hypothermic temperatures (Fig. 7). This causes more genotoxic stimuli (ROS) and hence more PARP activation at warm ischemia temperatures compared with colder ischemia temperatures associated with organ preservation. Therefore, an interplay between the salutary effects of slower PARP activity via its ability to accelerate the base excision repair system (27) and provide genomic stability vs. the harmful effects of excessive PARP activity with attendant energy collapse may dictate the overall biological effect that is observed (“good” vs. “bad”). Temperature during the ischemic period may tip the balance (by the degree of oxidant injury induced at reperfusion) as to which pathway predominates (Fig. 10).

Tubular injury induced by apoptosis at reperfusion after cold storage has been shown to contribute to preservation injury in renal proximal tubule epithelial cells (26) and may be exacerbated by PARP inhibition. Inhibition of PARP may be harmful by preventing the DNA repair process during hypothermia or rewarming, thus allowing apoptosis to initiate at reperfusion. If this occurs, it would proceed by a caspase-3-independent pathway because caspase-3 activity was not elevated with reperfusion in PARP inhibitor-treated renal proximal tubules, relative to the untreated controls (Table 1). However, some apoptotic pathways activated by genotoxic stimuli are independent of caspase-3 activation but instead rely on caspase-9 and p53 (11). Similar pathways may occur in cold-preserved renal tubules and run unchecked during PARP inhibition.

PARP inhibitors have been shown to dramatically attenuate reperfusion injury in warm models but not in models using cold ischemia. This was also demonstrated with PARP-1 knockout animals because PARP-1 deficiency did not protect isolated renal tubules from cold-storage reperfusion injury. However, unlike canine tubules in which PARP inhibitors potentiate preservation injury, inhibition of PARP synthesis by genetic knockout in the mouse did not. The difference between the canine results using PARP-1 inhibitors and the results in mice using genetic PARP-1 knockout is not clear but may be due to a decrease in the sensitivity of this described PARP effect in mice compared with dogs. This is further supported by the observation that PARP inhibition with PJ34 in wild-type control mice also did not potentiate LDH release after reperfusion. Experiments with isolated human renal proximal tubules isolated from kidneys that have been cold stored but not used for transplantation for technical reasons should indicate how human tissue behaves.

In conclusion, hypothermic ischemia and reperfusion of canine kidneys activate renal PARP activity. The effect requires reperfusion, is independent of oxygen toxicity, and is blocked by prior exposure to warm ischemia. PARP activation appears to play a protective role during hypothermic ischemia. Based on the current results, the inclusion of PARP inhibitors as a component of organ preservation fluids would be unlikely to be beneficial, or may even be deleterious.

GRANTS

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REFERENCES


